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12-12-2014	Final Report	13-Sep-2010 - 12-Sep-2014

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Final Report: Mechanism of UV-Induced Damage to Mammalian Collagen

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6. AUTHORS
Julian M Menter

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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720 Westview Dr., SW

Atlanta, GA 30310 -1458

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The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
The overall aim of this work has been to compare the effects of temperature on the rates and kinetics of the direct photochemical interaction between UV and mammalian collagen as functions of excitation wavelengths, temperature, fluorescence spectral distribution, and the presence of cellular environmental agents (e.g. dermal hyaluronic acid and molecular oxygen). Acid - soluble collagen extracted from 6 - 8 week old Skh - 1 hairless mice. Skh-1 collagen has a prominent band (excitation/emission = 270/360 nm; and involves molecular O2; shows 2nd order fading, but has little fluorescence at 325/400 nm (dityrosine). The 325/400 band INCREASES with UV

15. SUBJECT TERMS

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Report Title

Final Report: Mechanism of UV-Induced Damage to Mammalian Collagen

ABSTRACT

The overall aim of this work has been to compare the effects of temperature on the rates and kinetics of the direct photochemical interaction between UV and mammalian collagen as functions of excitation wavelengths, temperature, fluorescence spectral distribution, and the presence of cellular environmental agents (e.g. dermal hyaluronic acid and molecular oxygen). Acid - soluble collagen extracted from 6 - 8 week old Skh - 1 hairless mice. Skh-1 collagen has a prominent band (excitation/emission = 270/360 nm; and involves molecular O2; shows 2nd order fading, but has little fluorescence at 325/400 nm (dityrosine). The 325/400 band INCREASES with UV - irradiation and does not involve molecular O2. Experiments indicate that the 270/360 nm band slowly appears on "dry" samples at 4 C in the dark. Hyaluronate had a modest effect on the 270/360 nm kinetics. We found a reciprocal relationship between the 270/360 nm fading and the 325/400 nm build-up, suggesting the two prcesses are interrelated. Arrhenius plots of fading were distinctly non-linear, affording activation energies of ~ 0 kJ/mol at T < Tm and ~ 32 kJ/mol at T > Tm, consistent with consistent with a phase change near the melting temperature (~ 30 oC) requiring H-bond breakage

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Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received	Paper
12/16/2014 12.00	Julian M Menter, Amir Etemadi, Abrienne M Patta, Noah Scheinfeld. Topical AC-11 Abates While Applied Actinic Keratoses and Early Squamous Cell Cancers in Hairless Mice Exposed to Ultraviolet A (UVA) Radiation, APerito Journal of Dermatology, (12 2014): 102. doi:
12/17/2014 14.00	Julian M. Menter, Comnuan Nokkaew, Danita Eatman, Aquilla Sprewell, Natalia Silvestrov, Abrienne M. Patta, Sandra Harris-Hooker. The Role of Eumelanin in Generating Reactive Oxygen and Reactive Nitrogen in Solution: Possible Relevance to Keloid Formation, Open Journal of Physical Chemistry, (11 2013): 157. doi: 10.4236/ojpc.2013.34019
TOTAL:	**2**

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper
TOTAL:	

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Title: Effect of Temperature on Photochemical and Thermal Changes in Calf Skin Collagen Solutions at Physiological pH.

Julian M Menter, Latoya Freeman and Otega Edukuye, Morehouse School of Medicine, Atlanta, GA 30310-1495

Abstract

Mammalian collagens contain several age-related fluorescent chromophores derived from (photo)oxidation of tyrosine residues and/or glycation of free amino acid. Since these compounds may be photosensitizers or otherwise deleterious, it is important to know the chemical properties as well as the effects of temperature and the presence of oxygen on forming these age-related compounds under physiological conditions. Fluorescence be observed, whereas other properties (e.g. electrophoresis, appearance) may not be sensitive to age and temperature, probably because these fluorophores form a very small proportion of the collagen molecule. Deviation of Arrhenius plots from linearity suggests a change of phase and/or the simultaneous presence of more than one reaction. The non-linear plot observed in figure 3 is consistent with the collagen's helix-coil transition. Above Tm, the slopes of the measured fluorescence intensities are uncertain owing to the essentially random orientation so that estimated activation energies and values of Tm must be viewed as approximations. The data indicate that there is little or no activation involved in the photochemistry of the helical structure, There may be a small negative activation energy (fig 3B), indicating a possible "stable" region due to micro-unfolding near Tm (K. Kadler et al, J Biol. Chem. 263:10516 – 10523, 1988).The reciprocal relationship between the rate of 270/360 nm photo-degradation and consequent formation of stable dityrosine (excitation /emission at 325/400 nm) indicates that the two processes are interconnected. The 270/360 nm emission species appears to be a "double molecule". We have previously postulated this species to be due to an excimer - like interaction between two molecules in close proximity. However it could be due to covalent di-DOPA cross-links that disappear by UV- mediated formation of a non-fluorescent product. Our collagen samples were very hygroscopic and it was not possible for us to completely remove H2O. This allowed "dark" oxidation of tyrosine to DOPA. Our data thus indicate that di-DOPA may form via thermal oxidation of tyrosine at the latter's expense. This work was supported in part by DOD Grant # 911 NF-10-1, MBRS Grant # GM08248, and RCMI Grant # 8G12MD00760

To be presented at the BIT Molecular Medicine Conference Haikou, Hainan Province, P.R.China.

Pigment melanin mediates a redox reaction between adsorbed nitric oxide and O2 in vitro

J. Menter, C. Nokkaew. A. Sprewell and D. Eatman,
S. Harris-Hooker
Morehouse School or Medicine. Atlanta, GA. USA

Pigment melanin can adsorb molecular O2• scavenge nitric oxide (NO) and thereby coupie a redox reaction betvveen them. in this work. we show formation of peroxynitrite (ONOO-) in the presence but not in the absence of melanin. NO generated by DENNO or SNAP vvas dialyzed into membranes containIng purified sepia melanin in 0.1 M phosphate buffer, pH 7.4 or control buffer alone. NO was measured as nitrite and nitrate 'via the Greiss methodology and by the DAf fluorescence assay. Peroxynitrite vvas detected by selective scavenging with 3.3 fLM MCP or via detection of ni1rotyrosine in cultured fibroblasts. H:P2 w-'oS monitored by the scopoletirJperoxidase assay. Appropriate controls were used. Dialyzate NO concentrations were significantly lower in the test dialyzates than in controls. In the test systems in vitro we detected significant amounts of peroxynitrite but little Of no hydrogen peroxide. No significant amounts of either of these were detected in the absence of melanin. In cultured iibroblasts, we observed positive staining for nitrotyrosine in the presence, but not in the absence of melanin. Sepia melanin can couple the redox reaction between adsorbed NO ana O2 to afford ONOO - via a superoxide intermediate. Superoxide can undergo 'pseudodismutation' to H20 2 and O2 by melanin or reaction with NO. Peroxide is scavenged by melanin, and is not detected in significant amounts. Supported in Part by MBRS Grant ifGM 08248, RCMl Grant ifRR 03034 and DOD Grant it W911 NF - 10 - 1 - 0448.

IPCC, 2011International Pigment Cell Conference, Bordeaux, France, September 21 – 24, 2011

Peer – Reviewed Manuscript;

Julian M. Menter, Comnuan Nokkaew, Danita Eatman, Aquilla Sprewell1, Natalia Silvestrov Abrienne M. Patta, Sandra Harris-Hooker "The Role of Eumelanin in Generating Reactive Oxygen and Reactive Nitrogen in Solution: Possible Relevance to Keloid Formation" Open Journal of Physical Chemistry, 2013, 3, 157-162 Published Online November 2013 (http://www.scirp.org/journal/ojpc) http://dx.doi.org/10.4236/ojpc.2013.34019 Open Access OJPC

Acknowledgements This work was funded in part by GRANTS: MBRS #GM08248, RCMI #8G12MD007602, and DOD # 911 NF-10-1 0448. There are no conflicts of interest.

This manuscript will be sent as a separate

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received		Paper
08/28/2012	4.00	Latoya Freeman, Otega Edukuye, Julian Menter. Temperature and Age –Dependence of Type I Calf Skin Collagen in vitro, First Annual Morehouse School of Medicine Summser Experience for Medical Students. , . : ,
08/28/2013	7.00	Julian Menter. Collagen Fluorescence Spectral and Photochemical Behavior as Prognsticators of Skin Damage, BIT's Third Annual Conference on Biomarkers - 2012. 02-DEC-12, . : ,
TOTAL:	**2**	

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received		Paper
08/28/2012	2.00	AQUILLA SPREWELL, DANITA EATMAN, JULIAN MENTER, SANDRA HARRIS-HOOKER. Pigment melanin mediates a redox reaction between adsorbed nitric oxide and O2 in vitro, XXI ST INTERNATIONAL PIGMENT CELL CONFERENCE (IPCC). 20-SEP-11, . : ,
TOTAL:	**1**	

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received		Paper
09/02/2011	1.00	Julian Menter, Comnuan Nokkaew, Danita Eatman, Aquilla Sprewell. Pigment Melanin–Mediated Formation of Peroxynitrite from Nitric Oxide in Aerated Solutions and Fibroblast Cells In Vitro: Simultaneous Protective and Anti–Protective Behavior, Pigment Cell and Melanoma Research (09 2011)
TOTAL:	**1**	

Number of Manuscripts:

Books

Received	Book
TOTAL:	

Received	Book Chapter
TOTAL:	

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Post Doctorates

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Faculty Supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: .....

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):.....

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:.....

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense .....

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: .....

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

SCIENTIFIC GOAL – STATEMENT OF PROBLEM:

Virtually all studies on photo - aging have been concerned with wound – healing, as well as precancerous and cancerous sequelae that result from process far downfield from the primary interaction between UV photons and molecular collagen that ultimately lead to the observed deleterious effects. This approach has begged the question as to what are the molecular consequences of these interactions, and why the UV – irradiated collagen is treated as a foreign protein by the immune system. To address the latter question, we have embarked on a study of the direct photochemistry of mammalian collagen in solution/suspension at physiological pH. Since it is well known that the dermal milieu is more complicated than the “simple” solution,
we will also include some of these components, starting with hyaluronic acid (HA), the most abundant component of the interstitial gel.
Collagen has several covalently – bound fluorescent molecules that are unstable to solar UV wavelengths. Very little is known about their properties that affect their thermal and/or photostability.Even less is known as to their photoproducts, and whether or not these may be photosensitizers or possible phototoxic agents. Mammalian collagen has a very low turnover rate in vivo, so that such photo – modifications may pose a potential risk to society at large.

Summary of Most Important Results:

(1) We have carried out fluorescence transformation studies for the main collagen fluorophores. Most fruitful have been the 2nd order disappearance of the 270/360 nm(attributed to a disappearance of 2 like molecules; (either an eximer and/or a DOPA oxidation product) and the first order increase in 325/400 nm (attributable to dityrosine formation). The 270/360 nm species requires oxygen and is a “dark” reaction. This species accumulates on age even at 4o C. Dityrosine formation requires UV and does not accumulate in the dark. These two reactions have a “reciprocal relationship” that suggest that that the 270/360 nm species is formed at the expense of tyrosine.

(2) We have constructed Arrhenius Plots for fluorescence fading of the 270/360 nm species. Fading rates depend on the age and previous history (read “amount”) of the sample. Initial lack of sensitivity to this fact resulted in data that were not reliable, and led to the hypothesis that at moderate temperatures, there was a “stable region” that reflected micro-folding regions $\Delta E^* \sim 0$. In the last period, we were able to obtain better data. The activation energies (slope of the fading curve) were essentially zero from 8 – 30o C,although we could not rule out a small amount of micro-folding, since there was a slightly negative slope to the curve in this region (although it was not statistically
significant). Above the denaturation point, the data reflected the random nature of the coil conformation, as it gave a range of ΔE^* values ~ 8.8 ± 3.4 kcal/mol = 36.9 ± 14.1
kJ/mol (n = 3)

(3) We showed that melanin, ubiquitous to skin was able to couple (physiological) NO
oxidation to O2 reduction to generate ROS and RNS species. There may be a relationship
between this type of chemistry and keloid development. The chemical interaction(s) among melanin, NO, O2 and collagen remain essentially unknown.

Progress for the previous years are condensed form the original reports. These
are available on request.

YEAR 01: PERIOD: 13 September, 2010 – 31 July, 2011

The (original) specific aims of this project as outlined in the proposal are:

(1) Comparison of previously characterized purified type I acid soluble Skh – 1 hairless mouse collagen with collagen plus various amounts of added HA. Compare (a) measurement of rates and kinetics of photochemical reaction at several excitation wavelengths, as measured by fluorescence fading,
(b) temperature dependence studies of fluorescence
emission, and
(c) temperature dependence of photochemical fading.

(2) Test the extent to which observed collagen photochemical fading reactions depend on molecular O2. We will (a) run the UV photolysis in N2 – saturated solution, (b) test for generation of reactive oxygen species (ROS), as these been implicated in UV – induced photoaging and carcinogenesis. By comparing the results in the presence and absence of O2, ± ROS, we can obtain the relative importance of these to the direct collagen chemical photoreactions that lead to fluorescence fading.

Work with the less – soluble Skh – 1 hairless mouse collagen has been slower, primarily since we had to get approval for our animal protocols from our institution and from the DOD. For these and other reasons, we decided to suspend all animal studies and to concentrate on the calf – skin system. Highly purified commercial calf skin collagen (Elastin Products, Inc.Owensville MO) is readily available, and is soluble enough to use under physiological conditions.
We carried out UV photolysis with a UVG – 11 short wavelength hand lamp that emits primarily254 nm. We have obtained temperature – dependence fluorescence spectral and fading data from type I collagen (Coll) in the presence and absence of hyaluronate (HA) in a 1:2 (w/w) ratio.

Temperature Dependence of Collagen and Collagen – HA Fluorescence: The spectra and temperature – dependence of both collagen and collagen – HA (1:2) mixtures in 0.1 M phosphate buffer, pH = 7.4, were in close agreement with each other and in good agreement with the previously – published spectrum of calf – skin collagen in 0.05 M acetic acid (1).
Somewhat surprisingly, the presence of excess HA had virtually no effect on either parameter. Using a simple kinetic scheme (ref.1), we derived an approximate formula that is analogous to an Arrhenius plot. In a simple unbound molecule a plot of normalized reciprocal fluorescence, 1/ In, vs. reciprocal absolute temperature, 1 / T (oK) -1, will afford a straight line whose slope
affords an activation parameter, ΔE*, that is an approximation to the actual activation energy to within ~ 25%. Our plots are not straight lines, and this undoubtedly reflects the conformational change from helix – coil as the temperature is raised.

Temperature Dependence of Photochemical Fading of Collagen and Collagen – HA Mixtures:
The fading kinetics of calf – skin collagen ± HA are essentially different from those observed in our previous work (3) on acid – extracted Skh – 1 mouse collagen. In the mouse system, the fluorescence shows and excimer – like broad band emission at 360 nm which follows 2nd order fading kinetics. Calf skin Arrhenius plots were non-linear. In the mouse collagen, the species with 325/400 nm fluorescence was too weak for accurate measurements. However in the calf skin collagen, there was a measurable 325/400 nm band that increased as fading proceeded. This reaction was strongly retarded by the presence of HA, suggesting that polymer conformational change was necessary for the photochemical reaction (perhaps dityrosine formation?) to take place.
In the Arrhenius plots, the rate of formation of the 325/400 nm species showed a temperature dependence that could be sub – divided into two distinct temperature regions corresponding to helix (T < ~ 37 oC) and coil (T > ~ 37 oC). Above the denaturation point, the curves are not reproducible, but they depend on the physical state of the solution at the time of measurement. Above the denaturation point, the fluorescence build – up is significantly more rapid, and the activation parameter (read “activation energy”) is greater. This result is consonant with a requirement for the polymer to attain an optimal position for the reaction that produces an increase in the 325/400 nm fluorophore. Arrhenius Plots of Collagen (figure 3*; Black circles) and Collagen HA (figure 3*; White circles)
Afford a non – linear plot. Although there is a lot of scatter appears to be a relatively photo - stable temperature range from roughly 12oC – 35oC, with higher photolability outside these values. This finding is analogous to earlier results from several laboratories, in which conditions for de novo fibril assembly from was most favored at temperatures near body temperature. In these cases, the phenomenon was rationalized by the presence of intermediate micro – unfolded states at or near body temperature that facilitate fibril formation (K. Kadler et al, J Biol. Chem. 263(21) 10517 – 1063). However, (at the time of writing) these data are at present not reliable enough to draw any definite conclusions.

Generation of Reactive Oxygen Species (ROS) in Surrounding Melanin: We observed that sepia melanin, recognized as a good model for human eumelanin, can scavenge NO through a dialysis membrane in vitro. Melanin is an excellent electron transfer reagent and can also couple redox reactions that may produce or consume harmful radicals. Since melanin is a component of human dermis, it is possible that sunlight could form harmful melanin radicals that might
possibly degrade dermal collagen. In a manuscript supported in part by this grant we have detected formation of cytotoxic peroxynitrite (ONOO-) from physiological amounts of nitric oxide (NO) in the presence, but not in the absence of melanin.
Monitoring the extent of photolysis by viscosity measurements. Knowledge of the temperature and UV dependences of collagen ± HA will allow us to more accurately monitor the extent of
collagen and /or HA damages by UV, and they will shed more light on the photochemical results at temperatures higher than the denaturation point. We have, in fact, purchased two viscometers from Cannon instruments. Preliminary experiments indicate that it would be better to scale up the reaction, which would allow higher – bore viscometers that might increase the accuracy and precision of the experiments (low diameters are very slow and the solutions are
more susceptible to shear). We will correlate these viscosity measurements with
electrophoresis

Year 02 : Period 01 September, 2011 – 31 August, 2012
Aim (1) In the previous year we reported preliminary fluorescence fading data for calf –skin collagen and collagen – hyaluroniate (coll-HA) 1:2 mixtures, from 8.0oC ≤ T ≤ 62.0oC and constructed a preliminary Arrhenius Plot (see report for 31 August, 2012. Since then, we have concentrated more on the collagen system (“collagen alone”) and have obtained sufficiently better temperature dependence fading data to enable the 270/360 nm and the
325/400 fluorescence bands to afford interpretable results. Figure 1 shows our results thus far:

Aim (2) To date, we have conducted preliminary photolysis of collagen at several different temperatures in the presence and absence of molecular O2 (air). Air was excluded from the latter system by flushing a Thunberg cell, which served as a reaction vessel, with nitrogen gas followed by sealing with stopcock vacuum grease. The preliminary results at four temperatures ranging from 11.2 to 54.0oC indicated no significant effect of O2 on fluorescence fading. Unfortunately, we were not able to measure oxygen concentrations in
the cuvette, which left open the possibility that there was enough O2 in the nitrogen – flushed systems to interfere with the fading reaction even in the latter samples. These experiments will be carried on further in the third year from the latter system by flushing a Thunberg cell, which served as a reaction vessel, with nitrogen gas followed by sealing with stopcock vacuum

grease. The preliminary results at four temperatures ranging from 11.2 to
54.0oC indicated no significant effect of O2 on fluorescence fading. Unfortunately, we were not able to measure oxygen concentrations in the cuvette, which left open the possibility
that there was enough O2 in the nitrogen – flushed systems to interfere with the fading reaction even in the latter samples. These experiments will be carried on further in the third year.

Year 03 - 04: Period 01 September, 2012 – 31 August, 2014

This project was originally budgeted for 3 year. Because of the decision not to continue the animal experiments, we were able to extend the project for an extra year at no cost. In the meantime, with the help of 2 first year medical students, we undertook a more careful study of the effect of age on the fluorescence properties of calf skin collagen. We found a sample from
Elastin Products, Inc. that had sat in the dark at 4oC for ~ 5 years (Lot #121), and we found that its fluorescence spectrum was reminiscent of the Skh – 1 hairless albino mice that we had previously investigated (J M Menter, Photochem. Photobiol. Sci. 2006: 5, 403–410
DOI: 10.1039/b516429j; fig 2 below).

In addition to tyrosine fluorescence (λ max =; 275 nm excitation; 305 nm emission) figure 2
shows the presence of fluorophores resulting from post-translational thermal oxidation of tyrosine in extracted hairless mouse collagen viz. DOPA (285/325 nm), dityrosine (325/400 nm)“excimer-like interacting tyrosine residues in close proximity (?) and a weak shoulder at λ > 420
nm (DOPA oxidation products). These spectra may be compared with those of Lot #121 (> 5 years old) and a relatively new (at the time) Lot # 159.
The 270/360 nm fluorophore is photolabile to short wavelength UV, and the rate of fluorescence fading at 360 nm increases in proportion to “age”. One can see that some oxidation has taken place in the “newer” sample, Lot # 159.
One can rationalize the scattered fading data and consequent poorly - fitting Arrhenius plots reported previously by considering that the collagen solutions used sat over a significant period of time in buffered solution and even the “dry” collagen samples slowly oxidized. Thus, a “moving target” that we were insensitive to was in force.
The opposite effect occurred for the 325/400 nm data. The fluorescence build - up of this species (dityrosine) ensued fastest when the [DOPA]
oxidation products were lowest (i.e with collagen that had not aged significantly)

Lot # 159 (light circles). The rate of fluorescence build-up is greater in the
dearth or absence of DOPA oxidation product. This shows that the opposite effect occurs for the 325/400 nm data. We were able to obtain a “new” collagen sample (Lot # 267) whose fluorescence excitation and emission spectra were very similar to nascent collagen, which contains only tyrosine. (figure 6).
The 270/360 nm pair, not very prominent in the fluorescence spectrum
is non-linear, and that the 325/400 nm pair is fades approximately as rapidly that in Lot# 159.

ARRHENIUS PLOTS; UPDATED.
Awareness that the rate of fading of the 270/360 nm fluorescence pair critically depends on the age and previous history of the sample being analyzed has led us to do additional experiments where, as far as practical, the age of the sample has been kept more or less constant. Figure 8a shows that the resulting Arrhenius plot seems to indicate that below the denaturation point the plots are essentially flat. However, the correlation coefficient is poor. (r2 = 0.019)

Although the fading at T < 30o C (330 oK) seems to indicate a “flat” slope (i.e. no activation energy) the data in this region are still not precise enough to warrant a definite conclusion.

Therefore, another set of experiments were carried for temperatures ranging from 8 – 25 o C,
using fresh collagen samples. The results are consistent with Ea = 0, but there may be a slightly negative slope indicating that there may be a small amount of
stabilization due to micro melting of the helical superstructure (n = 3)
slope indicating that there may be a small amount of stabilization due to micro melting of the helical superstructure (n = 3)

Effect of pigment melanin on generation of ROS and RNS:
Julian M. Menter1, Comnuan Nokkaew2, Danita Eatman3, Aquilla Sprewell1, Natalia
Silvestrov3, Abrienne M. Patta1, Sandra Harris-Hooker2 Open Journal of Physical Chemistry,
2013, 3, 157-162 Published Online November 2013 (http://www.scirp.org/journal/ojpc)
http://dx.doi.org/10.4236/ojpc.2013.34019. Recently, nitric oxide (NO) has been implicated as an epigenetic factor in keloids, a scarring disease occurring primarily in dark skinned people who have relatively high amounts of pigment melanin. In this work, we tested whether a melanin- mediated redox reaction involving adsorbed NO and O2 can couple NO oxidation with O2 reduction to form reactive oxygen species (ROS) or reactive nitrogen species (RNS) in vitro at pH 7.4. We measured the formation of reactive species that oxidize dihydrorhodamine123 (DHR)to fluorescent rhodamine123 in the presence and

absence of sepia melanin. In separate experiments, we monitored NO concentration with 4,5-diaminofluorescein (DAF) by measuring
the highly fluorescent NO-adduct, DAF-2T. We attempted to detect peroxynitrite with 5 μM 3- methyl-1,2-cyclopentanedione (MCP), a selective scavenger of peroxynitrite (IC50 = 3.6 μM for ONOO- vs. 63.8 μM and >> 100 μM for NO and respectively). However, MCP itself oxidized DHR. We found that in the absence of NO, melanin itself oxidizes DHR, with no loss of DAF fluorescence
(i.e. no net consumption of NO). In the presence of NO, there was a ~57% loss of
DAF fluorescence, indicating that NOx is formed at the expense of NO. The data provided good fit (r2 = 0.94) to a Langmuir adsorption isotherm, with pseudo first order rate k' = 8.2 × 107 s-1 and adsorption coefficient Kad = 4.04 M-1. Both of these parameters are consistent with a facile chemisorption reaction between NO and O2 on the melanin surface. Possible reactions are a)
NO and O2 ◊ ONOO- and/or b) 2NO + O2 ◊ 2NO2. The latter reaction is disfavored in solution but is significantly accelerated on the melanin surface via an entropy effect.

Summary of Most Important Results:
(1) We have carried out fluorescence transformation studies for the main collagen fluorophores. Most fruitful have been the 2nd order disappearance of the 270/360 nm (attributed to a disappearance of 2 like molecules; (either an eximer and/or a DOPA oxidation product) and the first order increase in 325/400 nm (attributable to dityrosine formation). The 270/360 nm species requires oxygen and is a “dark” reaction. This species accumulates on age even at 4o C. Dityrosine formation requires UV and does not accumulate in the dark. These two reactions have a “reciprocal relationship” that suggest that that the 270/360 nm species is formed at the expense of tyrosine.

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Fading rates depend on the age and previous history (read “amount”) of the sample. Initial lack of sensitivity to this fact resulted in data that were not reliable, and led to the hypothesis that at moderate temperatures, there was a “stable region” that reflected micro-folding regions ΔE*~ 0. In the last period, we were able to obtain better data. The activation energies (slope of the fading curve) were essentially zero from 8 – 30o C,although we could not rule out a small amount of micro-folding, since there was a slightly negative slope to the curve in this region (although it was not statistically
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kJ/mol (n = 3)

Technology Transfer

REPORT DOCUMENTATION PAGE

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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
31-10-2014	FINAL REPORT	13 SEPT 2010 - 12 SEPT 2014

4. TITLE AND SUBTITLE

"Mechanism of UV-Induced Damage to Mammalian Collagen"

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W911NF-10-1-0448

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

JULIAN M MENTER, PHD

5d. PROJECT NUMBER

5e. TASK NUMBER

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14. ABSTRACT

The overall aim of this work has been to compare the effects of temperature on the rates and kinetics of the direct photochemical interaction between UV and mammalian collagen as functions of excitation wavelengths, temperature, fluorescence spectral distribution, and the presence of cellular environmental agents (e.g. dermal hyaluronic acid and molecular oxygen). Acid - soluble collagen extracted from 6 - 8 week old Skh - 1 hairless mice. Skh-1 collagen has a prominent band (excitation/emission = 270/360 nm; and involves molecular O2; shows 2nd order fading, but has little fluorescence at 325/400 nm (dityrosine). The 325/400 band INCREASES with UV - irradiation and does not involve molecular O2. Experiments indicate that the 270/360 nm band slowly appears on "dry" samples at 4 C in the dark. Hyaluronate had a modest effect on the 270/360 nm kinetics. We found a reciprocal relationship between the 270/360 nm fading and the 325/400 nm build-up, suggesting the two prcesses are interrelated. Arrhenius plots of fading were distinctly non-linear, affording activation energies of ~ 0 kJ/mol at T < Tm and ~ 32 kJ/mol at T > Tm, consistent with consistent with a phase change, near the melting temperature (~ 30 oC) requiring H-bond breakage.

15. SUBJECT TERMS

Mammalian Collagens, Fluorescence Spectra, Fluorescence Fading, Photochemical Kinetics, Thermal ("dark") Reactions, Age-related changes.

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MOREHOUSE SCHOOL OF MEDICINE

PRINCIPAL INVESTIGATOR (PI)

JULIAN MENTER

CONTRACT NO.

W911NF-10-1-0448

AWARD TITLE

“MECHANISM OF UV-INDUCED DAMAGE TO MAMMALIAN COLLAGEN”

PERIOD OF PERFORMANCE

9/13/2010 – 9/12/2014

FINAL REPORT

FINAL PROGRESS REPORT – W911NF-10-0448
"MECHANISM OF UV DAMAGE TO MAMMALIAN COLLAGEN"
JULIAN M MENTER, PROJECT DIRECTOR.

PERIOD: 13 SEPT 2010 - 12 SEPT 2014

FORWARD:

Mammalian Type I calf - skin collagen has fluorescent one or more compounds on their telopeptides (non-helical regions). The number and structure of these fluorophores vary with the age and previous history of a given sample. These fluorophores have been identified as tyrosine (excitation at 275, emission at 305 nm, dihydroxyphenylalanine (DOPA) 280/325 nm; dityrosine (325/410 nm); DOPA oxidation products, namely "excimer – like "double molecule" (285/360 nm (see report) and 370/450 nm (probably a mixture).

These molecules are photolabile to solar wavelengths, which raises the question as to whether the resulting photochemical reactions and/or photoproducts are deleterious to collagen or other molecules in the vicinity. Direct photolysis of collagen causes fluorescence fading in some cases, and buildup of the 325/400 nm (dityrosine). In addition, these molecules are also thermally unstable, and can "spontaneously" oxidize, even in the dark at 4 ^{o} C

The original purpose of this study has been to study the temperature dependence of the photochemical kinetics of fluorescence fading and/or buildup under 254 nm UV. This has been expanded to include (1) the above-mentioned "dark" transformations and (2) the ability of pigment melanin to couple oxidation of ubiquitous nitric oxide (NO) with oxygen reduction to form reactive oxygen species (ROS) and reactive nitrogen species (RNS). This progress report describes these experiments.

Although this grant has ended, work on this problem is still on-going. We will keep the DOD abreast of the progress in the future.

TABLE OF CONTENTS:

APPENDIX

(a) Papers Published in Peer – Reviewed Journals

Julian M. Menter, Comnuan Nokkaew, Danita Eatman, Aquilla Sprewell, Natalia Silvestrov, Abrienne M. Patta, Sandra Harris-Hooker (2013). The Role of Eumelanin in Generating Reactive Oxygen and Reactive Nitrogen in Solution: Possible Relevance to Keloid Formation. ***Open Journal of Physical Chemistry*, 2013, 3, 157-162** (http://www.scirp.org/journal/ojpc) http://dx.doi.org/10.4236/ojpc.2013.34019 Open Access ***OJPC (WILL BE SENT AS SEPARATE ATTACHMENT)***

(b) TO BE PRESENTED:

Title: *Effect of Temperature on Photochemical and Thermal Changes in Calf Skin Collagen Solutions at Physiological pH.*

Julian M Menter, Latoya Freeman and Otega Edukuye, Morehouse School of Medicine, Atlanta, GA 30310-1495

Abstract

Mammalian collagens contain several age-related fluorescent chromophores derived from (photo)oxidation of tyrosine residues and/or glycation of free amino acid. Since these compounds may be photosensitizers or otherwise deleterious, it is important to know the chemical properties as well as the effects of temperature and the presence of oxygen on forming these age-related compounds under physiological conditions. Fluorescence be observed, whereas other properties (e.g. electrophoresis, appearance) may not be sensitive to age and temperature, probably because these fluorophores form a very small proportion of the collagen molecule. Deviation of Arrhenius plots from linearity suggests a change of phase and/or the simultaneous presence of more than one reaction. The non-linear plot observed in *figure 3* is consistent with the collagen's helix-coil transition. Above *Tm*, the slopes of the measured fluorescence intensities are uncertain owing to the essentially random orientation so that estimated activation energies and values of *Tm* must be viewed as approximations. The data indicate that there is little or no activation involved in the photochemistry of the helical structure, There may be a small negative activation energy (*fig 3B*), indicating a possible "stable" region due to micro-unfolding near *Tm* (*K. Kadler et al, J Biol. Chem. 263:10516 – 10523, 1988).*The reciprocal relationship between the rate of 270/360 nm photo-degradation and consequent formation of stable dityrosine (excitation /emission at 325/400 nm) indicates that the two processes are interconnected. The 270/360 nm emission species appears to be a "double molecule". We have previously postulated this species to be due to an excimer - like interaction between two molecules in close proximity. However it could be due to covalent di-DOPA cross-links that disappear by UV- mediated formation of a non-fluorescent product. Our collagen samples were very hygroscopic and it was not possible for us to completely remove H2O. This allowed "dark" oxidation of tyrosine to DOPA. Our data thus indicate that di-DOPA may form via thermal oxidation of tyrosine at the latter's expense. **This work was supported in part by DOD Grant # 911 NF-10-1, MBRS Grant # GM08248, and RCMI Grant # 8G12MD00760**

To be presented at the BIT Molecular Medicine Conference Haikou, Hainan Province, P.R.China.

(1) CONTENT: STATEMENT OF PROBLEM:

The specific aims of this project as outlined in the proposal were:

(1) Comparison of previously characterized purified type I acid soluble Skh – 1 hairless mouse collagen with type I calf skin collagen ± added HA. The latter system more closely approximates the *in vivo* dermal milieu. Compare (a) *measurement of rates and kinetics of photochemical reaction at several excitation wavelengths, as measured by fluorescence fading*, (b) *temperature dependence studies of fluorescence emission,* and (c) *temperature dependence of photochemical fading.*

(2) Test the extent to which observed collagen photochemical fading reactions depend on molecular O_2. We will (a) run the UV photolysis in N_2 – saturated solution, (b) test for generation of reactive oxygen species (ROS), as these been implicated in UV – induced photoaging and carcinogenesis. By comparing the results in the presence and absence of O_2, ± ROS, we can obtain the relative importance of these to the direct collagen chemical photoreactions that lead to fluorescence fading.

During the ensuing grant period we have expanded these aims to include the effects of thermal reactions on these fluorescence alterations in calf skin type I collagen (aim 1) and the role of pigment melanin on the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Aim 2). The latter study has been initiated in part as a result of our previous preliminary study (JM Menter et al, Research Letters in Physical Chemistry Volume 2008, Article ID 210616, 4 pages doi:10.1155/2008/210616) that indicates that melanin binds molecular O_2. We hypothesized that physiological nitric oxide (NO), present near dermal blood vessels, might also bind to melanin and create a redox system that would simultaneously reduce adsorbed oxygen and oxidize NO to generate both ROS and RNS. Here we present evidence that such is actually the case (see below). As melanin is ubiquitous, there is a good chance that it might interact in some way with dermal collagen. A study of this effect would bring increased perspective on the way(s) that collagen would react to heat and light. This type of insight would be of great use to the Army in gauging risk/benefits involved to soldiers and other personnel when working in extreme climates

SCIENTIFIC GOAL – STATEMENT OF PROBLEM:

Virtually all studies on photo - aging have been concerned with wound – healing, as well as precancerous and cancerous sequelae that result from process far downfield from the primary interaction between UV photons and molecular collagen that ultimately lead to the observed deleterious effects. This approach has begged the question as to *what* are the molecular consequences of these interactions, and *why* the UV – irradiated collagen is treated as a foreign protein by the immune system. To address the latter question, we have embarked on a study of the *direct* photochemistry of mammalian collagen in solution**/**suspension at physiological pH. Since it is well known that the dermal milieu is more complicated than the "simple" solution, we will also include some of these components, starting with hyaluronic acid (HA), the most abundant component of the interstitial gel.

Collagen has several covalently – bound fluorescent molecules that are unstable to solar UV wavelengths. Very little is known about their properties that affect their thermal and/or photo-stability. Even less is known as to their photoproducts, and whether or not these may be photosensitizers or possible phototoxic agents. Mammalian collagen has a very low turnover rate *in vivo*, so that such photo – modifications may pose a potential risk to society at large.

Summary of Most Important Results:

(1) We have carried out fluorescence transformation studies for the main collagen fluorophores. Most fruitful have been the 2nd order ***disappearance*** of the 270/360 nm (attributed to a disappearance of 2 like molecules; (either an eximer and/or a DOPA oxidation product) and the first order ***increase*** in 325/400 nm (attributable to dityrosine formation). The 270/360 nm species requires oxygen and is a "dark" reaction. This species accumulates on age even at 4^{o} C. Dityrosine formation requires UV and does not accumulate in the dark. These two reactions have a "reciprocal relationship" that suggest that that the 270/360 nm species is formed at the expense of tyrosine.

(2) We have constructed Arrhenius Plots for fluorescence fading of the 270/360 nm species. Fading rates depend on the age and previous history (read "amount") of the sample. Initial lack of sensitivity to this fact resulted in data that were not reliable, and led to the hypothesis that at moderate temperatures, there was a "stable region" that reflected micro-folding regions $\Delta E^* \sim 0$. In the last period, we were able to obtain better data. The activation energies (slope of the fading curve) were essentially zero from 8 – 30^{o} C, although we could not rule out a small amount of micro-folding, since there was a slightly negative slope to the curve in this region (although it was not statistically significant). Above the denaturation point, the data reflected the random nature of the coil conformation, as it gave a *range* of ΔE^* values ~ 8.8 ± 3.4 kcal/mol = 36.9 ± 14.1 kJ/mol (n = 3)

(3) We showed that melanin, ubiquitous to skin was able to couple (physiological) NO oxidation to O_2 reduction to generate ROS and RNS species. There may be a relationship between this type of chemistry and keloid development. The chemical interaction(s) among melanin, NO, O_2 and collagen remain essentially unknown.

Progress for the previous years are condensed form the original reports. These are available on request.

YEAR 01: PERIOD: 13 September, 2010 – 31 July, 2011

The (original) specific aims of this project as outlined in the proposal are:

(1) Comparison of previously characterized purified type I acid soluble Skh – 1 hairless mouse collagen with collagen plus various amounts of added HA. Compare (a) measurement of rates and kinetics of photochemical reaction at several excitation wavelengths, as measured by fluorescence fading, (b) temperature dependence studies of fluorescence emission, and (c) temperature dependence of photochemical fading.

(2) Test the extent to which observed collagen photochemical fading reactions depend on molecular O2. We will (a) run the UV photolysis in N2 – saturated solution, (b) test for generation of reactive oxygen species (ROS), as these been implicated in UV – induced photoaging and carcinogenesis. By comparing the results in the presence and absence of O_2, ± ROS, we can obtain the relative importance of these to the direct collagen chemical photoreactions that lead to fluorescence fading.

Work with the less – soluble Skh – 1 hairless mouse collagen has been slower, primarily since we had to get approval for our animal protocols from our institution and from the DOD. For these and other reasons, we decided to suspend all animal studies and to concentrate on the calf – skin system. Highly purified commercial calf skin collagen (Elastin Products, Inc. Owensville MO) is readily available, and is soluble enough to use under physiological conditions. We carried out UV photolysis with a UVG – 11 short wavelength hand lamp that emits primarily 254 nm. We have obtained temperature – dependence fluorescence spectral and fading data from type I collagen (Coll) in the presence and absence of hyaluronate (HA) in a 1:2 (w/w) ratio. Temperature Dependence of Collagen and Collagen – HA Fluorescence: The spectra and temperature – dependence of both collagen and collagen – HA (1:2) mixtures in 0.1 M phosphate buffer, pH = 7.4, were in close agreement with each other and in good agreement with the previously – published spectrum of calf – skin collagen in 0.05 M acetic acid (1). Somewhat surprisingly, the presence of excess HA had virtually no effect on either parameter.

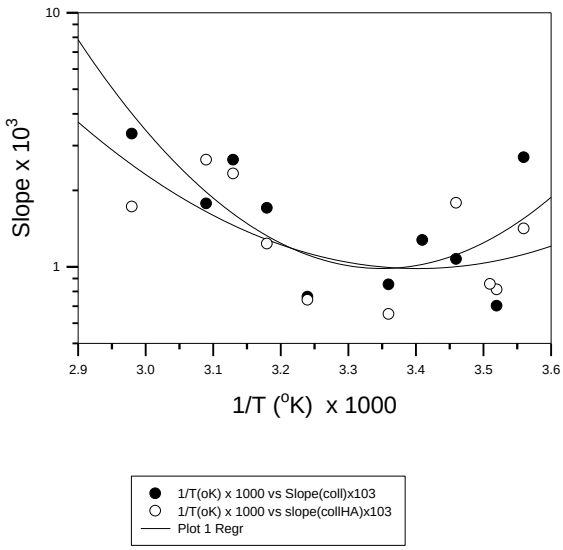
Using a simple kinetic scheme (ref.1), we derived an approximate formula that is analogous to an Arrhenius plot. In a simple unbound molecule a plot of normalized reciprocal fluorescence, 1 / In, vs. reciprocal absolute temperature, 1 / T (oK) -1, will afford a straight line whose slope affords an activation parameter, ΔE*, that is an approximation to the actual activation energy to within ~ 25%. Our plots are not straight lines, and this undoubtedly reflects the conformational change from helix – coil as the temperature is raised.

Temperature Dependence of Photochemical Fading of Collagen and Collagen – HA Mixtures: The fading kinetics of calf – skin collagen ± HA are essentially different from those observed in our previous work (3) on acid – extracted Skh – 1 mouse collagen. In the mouse system, the fluorescence shows and excimer – like broad band emission at 360 nm which follows 2nd order fading kinetics. Calf skin Arrhenius plots were non-linear. In the mouse collagen, the species with 325/400 nm fluorescence was too weak for accurate measurements. However in the calf skin collagen, there was a measurable 325/400 nm band that *increased* as fading proceeded. This reaction was strongly retarded by the presence of HA, suggesting that polymer conformational change was necessary for the photochemical reaction (perhaps dityrosine formation?) to take place.

In the Arrhenius plots, the rate of formation of the 325/400 nm species showed a temperature dependence that could be sub – divided into two distinct temperature regions corresponding to helix (T < ~ 37 oC) and coil (T > ~ 37 oC). Above the denaturation point, the curves are not reproducible, but they depend on the physical state of the solution at the time of measurement. Above the denaturation point, the fluorescence build – up is significantly more rapid, and the activation parameter (read “activation energy”) is greater. This result is consonant with a requirement for the polymer to attain an optimal position for the reaction that produces an increase in the 325/400 nm fluorophore.

Arrhenius Plots of Collagen (*figure 3*;* Black circles) and Collagen HA (*figure 3*;* White circles) Afford a non – linear plot. Although there is a lot of scatter appears to be a relatively photo - stable temperature range from roughly $12^{o}C$ – $35^{o}C$, with higher photolability outside these values. This finding is analogous to earlier results from several laboratories, in which conditions for *de novo* fibril assembly from was most favored at temperatures near body temperature. In these cases, the phenomenon was rationalized by the presence of intermediate micro – unfolded states at or near body temperature that facilitate fibril formation **(*K. Kadler et al, J Biol. Chem. 263(21) 10517 – 1063).*** However, (at the time of writing) these data are at present not reliable enough to draw any definite conclusions.

Figure 3
Arrhenius Plot of Collagen and Collagen HA



*The figure numbers correspond to those in the previous reports

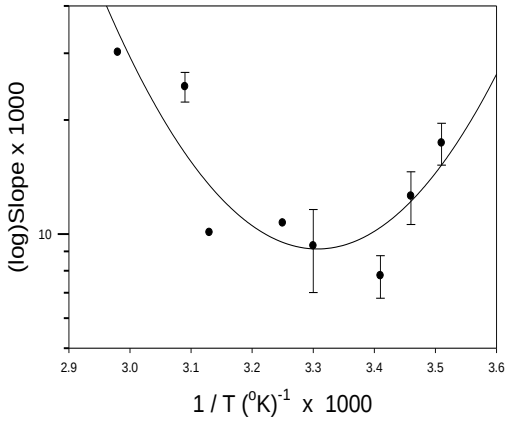
Generation of Reactive Oxygen Species (ROS) in Surrounding Melanin: We observed that sepia melanin, recognized as a good model for human eumelanin, can scavenge NO through a dialysis membrane in vitro. Melanin is an excellent electron transfer reagent and can also couple redox reactions that may produce or consume harmful radicals. Since melanin is a component of human dermis, it is possible that sunlight could form harmful melanin radicals that might possibly degrade dermal collagen. In a manuscript supported in part by this grant we have detected formation of cytotoxic peroxynitrite (ONOO-) from physiological amounts of nitric oxide (NO) in the presence, but not in the absence of melanin.

Monitoring the extent of photolysis by viscosity measurements. Knowledge of the temperature and UV dependences of collagen ± HA will allow us to more accurately monitor the extent of collagen and /or HA damages by UV, and they will shed more light on the photochemical results at temperatures higher than the denaturation point. We have, in fact, purchased two viscometers from Cannon instruments. Preliminary experiments indicate that it would be better to scale up the reaction, which would allow higher – bore viscometers that might increase the accuracy and precision of the experiments (low diameters are very slow and the solutions are more susceptible to shear). We will correlate these viscosity measurements with electrophoresis

Year 02 : Period 01 September, 2011 – 31 August, 2012

Aim (1) In the previous year we reported preliminary fluorescence fading data for calf –skin collagen and collagen – hyaluroniate (coll-HA) 1:2 mixtures, from $8.0^{o}C \leq T \leq 62.0^{o}C$ and constructed a preliminary Arrhenius Plot (see report for 31 August, 2012. Since then, we have concentrated more on the collagen system ("collagen alone") and have obtained sufficiently better temperature dependence fading data to enable the 270/360 nm and the 325/400 fluorescence bands to afford interpretable results. Figure 1 shows our results thus far:

Figure 1:



Arrhenius plot of calf skin collagen fading under 254 nm UV. Slope of curves are proportional to molecular rate constant for disappearance of the 270/360 nm band. Error bars reflect results from at least 3 separate experiments.

Experiments above Tm (~ 323^{o}K) (n=4) yielded results that varied from 7 – 15 kcal/ mole. This reflects the "randomness" of the coiled form.

The present results more clearly seem to indicate the existence of a "stable region" (~ 20 – 30^{o} C) in fluorescence fading is slowest. Below this region (T < 20^{o}C) there is a negative activation energy. By dividing the data of figure 1 into 3 quasi – linear curves in the "low" (T < 20^{o} C) "middle" (20 < T < 30^{o}C) and "high" (T > Tm), we were able to estimate these activation energies as ΔE_{low} = -15.6 kcal/mol (-65.2 kJ/mol); ΔE_{mid} ~ 0; ΔE_{high} = 9.70 kcal/mol (41 kJ/mol).

Aim (2) To date, we have conducted preliminary photolysis of collagen at several different temperatures in the presence and absence of molecular O_2 (air). Air was excluded Aim (2) To date, we have conducted preliminary photolysis of collagen at several different temperatures in the presence and absence of molecular O2 (air). Air was excluded from the latter system by flushing a Thunberg cell, which served as a reaction vessel, with nitrogen gas followed by sealing with stopcock vacuum grease. The preliminary results at four temperatures ranging from 11.2 to 54.0oC indicated no significant effect of O2 on fluorescence fading. Unfortunately, we were not able to measure oxygen concentrations in the cuvette, which left open the possibility that there was enough O2 in the nitrogen – flushed systems to interfere with the fading reaction even in the latter samples. These experiments will be carried on further in the third year from the latter system by flushing a Thunberg cell, which served as a reaction vessel, with nitrogen gas followed by sealing with stopcock vacuum grease. The preliminary results at four temperatures ranging from 11.2 to 54.0^{o}C indicated no significant effect of O_2 on fluorescence fading. Unfortunately, we were not able to measure oxygen concentrations in the cuvette, which left open the possibility that there was enough O_2 in the nitrogen – flushed systems to interfere with the fading reaction even in the latter samples. These experiments will be carried on further in the third year.

Year 03 - 04: Period 01 September, 2012 – 31 August, 2014

This project was originally budgeted for 3 year. Because of the decision not to continue the animal experiments, we were able to extend the project for an extra year at no cost. In the meantime, with the help of 2 first year medical students, we undertook a more careful study of the effect of age on the fluorescence properties of calf skin collagen. We found a sample from Elastin Products, Inc. that had sat in the dark at 4^{o}C for ~ 5 years (Lot #121), and we found that its fluorescence spectrum was reminiscent of the Skh – 1 hairless albino mice that we had previously investigated **(J M Menter, *Photochem. Photobiol. Sci*. 2006: *5*, 403–410 DOI: 10.1039/b516429j; *fig 2* below).**

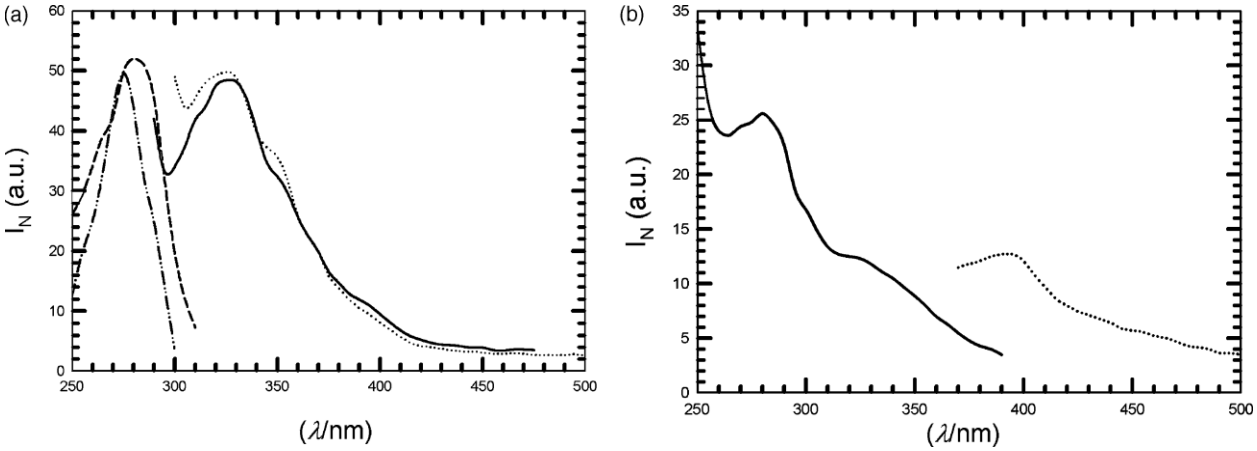


Figure 2: (a) *Fluorescence excitation and emission spectra of* ***Skh-1 acid-soluble hairless mouse collagen (0.25 mg ml−1 in 0.05 M HOAc).*** *Solid line: fluorescence excited at 270 nm; dotted line: fluorescence excited at 285 nm; dot dashed line: excitation of 300 nm fluorescence; dashed line: excitation of 360 nm fluorescence****. (b)*** *Dotted line: fluorescence excited at 325 nm; solid line: excitation of 430 nm fluorescence. Excitation of fluorescence at 450 nm gave rise to a very weak band at ca. 370 nm.*

In addition to tyrosine fluorescence (λ max =; 275 nm *excitation;* 305 nm *emission*) figure 2 shows the presence of fluorophores resulting from post-translational thermal oxidation of tyrosine in extracted hairless mouse collagen *viz.* DOPA (285/325 nm), dityrosine (325/400 nm) "excimer-like interacting tyrosine residues in close proximity (?) and a weak shoulder at λ > 420 nm (DOPA oxidation products).

These spectra may be compared with those of Lot #121 (> 5 years old) and a relatively new (at the time) Lot # 159.

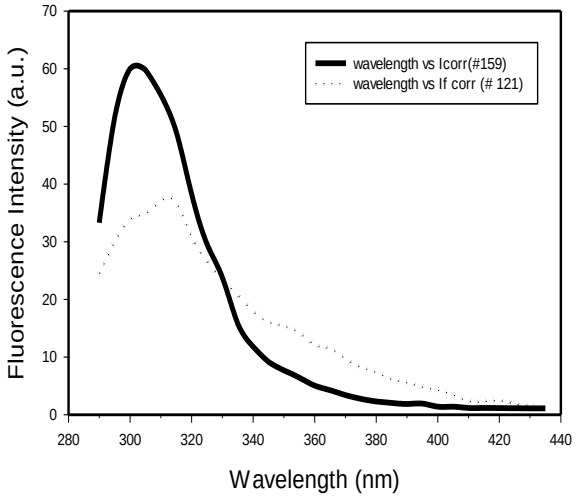


Fig. 3. Fluorescence emission spectra of ***Calf Skin Type I Collagen (Elastin Products, Inc.)*** *Solid line: Sample Lot # 159; Dotted line Sample Lot # 121. Excitation λ = 270 nm.*

Clearly, oxidation of tyrosine and subsequent DOPA oxidation products is a "dark" reaction that occurs even at 4 ^{o}C.

The 270/360 nm fluorophore is *photo*labile to short wavelength UV, and the rate of fluorescence fading at 360 nm increases in proportion to "age". One can see that some oxidation has taken place in the "newer" sample, Lot # 159. Figure 4 shows that the rate of 2nd- order fading is therefore age-dependent.

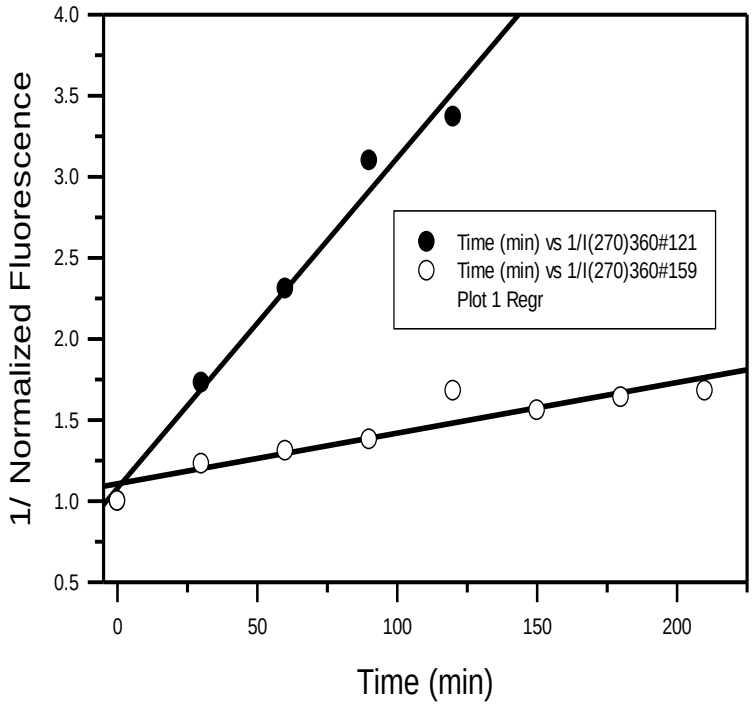


Figure 4. Rate of second-order fading of 270/360 nm fluorescence for Lot # 121 (black circles) and Lot # 159 (light circles).

One can rationalize the scattered fading data and consequent poorly - fitting Arrhenius plots reported previously by considering that the collagen solutions used sat over a significant period of time in buffered solution and even the "dry" collagen samples slowly oxidized. Thus, a "moving target" that we were insensitive to was in force.

Figure 5 shows that the opposite effect occurred for the 325/400 nm data. The fluorescence build - up of this species (dityrosine) ensued fastest when the [DOPA] oxidation products were lowest (i.e with collagen that had not aged significantly)

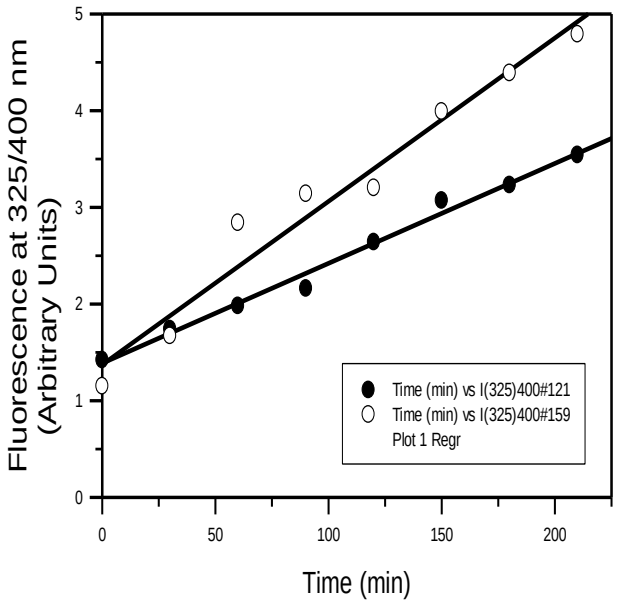


*Figure 5 Rate of build-up of 325/400 nm fluorescence for Lot # 121 (black circles) and Lot # 159 (light circles). Note that the rate of fluorescence build-up is **greater** in the dearth or absence of DOPA oxidation product. This shows that the opposite effect occurs for the 325/400 nm data.*

We were able to obtain a "new" collagen sample (Lot # 267) whose fluorescence excitation and emission spectra were very similar to nascent collagen, which contains only tyrosine *(figure 6).*

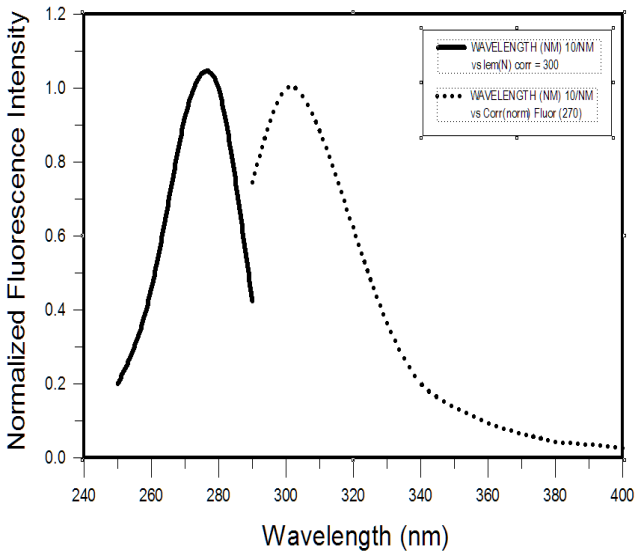


Figure 6: *Fluorescence excitation (solid line) and emission (dotted line) of newly – obtained Lot # 267. Note the absence of tyrosine oxidation products.*

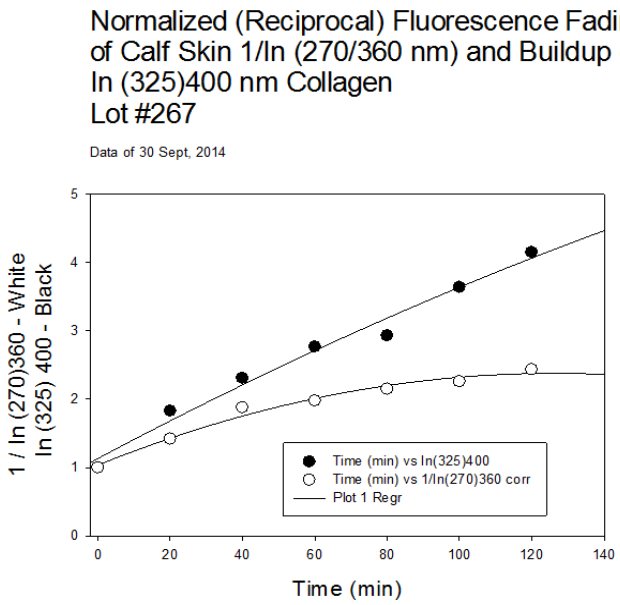


Figure 7: Normalized fluorescence transformation curves of calf skin collagen ***Lot # 267****. Black circles: Build-up of In 325/400 nm fluorescence; white curves: fading of 1 /In 270/360 nm fluorescence. Notice that the 270/360 nm pair, not very prominent in the fluorescence spectrum is non-linear, and that the 325/400 nm pair is fades approximately as rapidly that in Lot# 159.*

ARRHENIUS PLOTS; UPDATED.

Awareness that the rate of fading of the 270/360 nm fluorescence pair critically depends on the age and previous history of the sample being analyzed has led us to do additional experiments where, as far as practical, the age of the sample has been kept more or less constant. *Figure 8a* shows that the resulting Arrhenius plot seems to indicate that below the denaturation point the plots are essentially flat. However, the correlation coefficient is poor. ($r^2 = 0.019$)

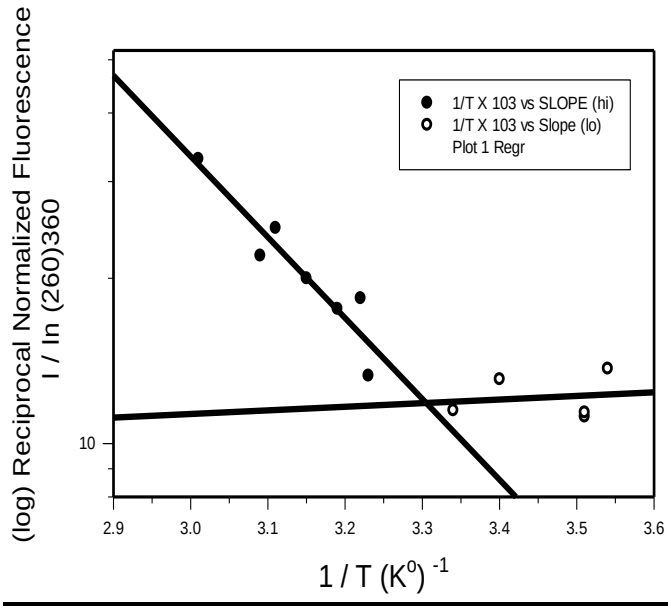


Figure 8a *Recent Arrhenius plot for fluorescence fading of the 270/360 nm pair (Lot #121). Although the fading at T < 30o C (330 ^{o}K) seems to indicate a "flat" slope (i.e. no activation energy) the data in this region are still not precise enough to warrant a definite conclusion (see text).*

Therefore, another set of experiments were carried for temperatures ranging from 8 – 25 ^{o} C, using fresh collagen samples. The results are shown in *figure 8b*. This plot is consistent with Ea = 0, but there may be a slightly negative slope indicating that there may be a small amount of stabilization due to micro melting of the helical superstructure **(n = 3)**

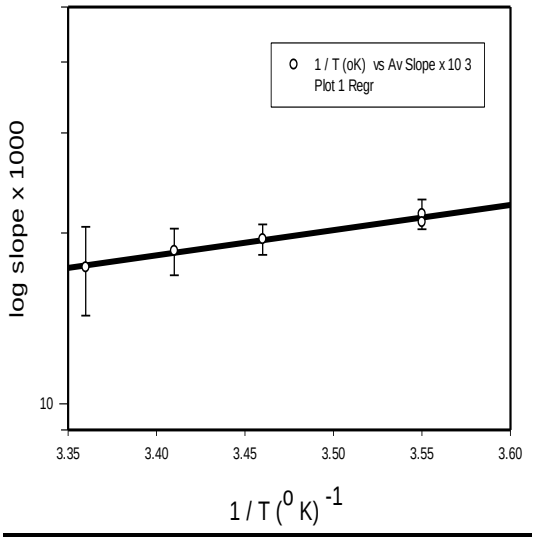


Figure 8b. *Updated Arrhenius Plot for fluorescence fading of the 270/360 nm pair in the region 8.0 – 25.0 ^{o} C. This plot is consistent with Ea = 0, but there may be a slightly negative slope indicating that there may be a small amount of stabilization due to micro melting of the helical superstructure* ***(n = 3)***

<u>Effect of pigment melanin on generation of ROS and RNS:</u>

Julian M. Menter[1], Comnuan Nokkaew[2], Danita Eatman[3], Aquilla Sprewell[1], Natalia Silvestrov[3], Abrienne M. Patta[1], Sandra Harris-Hooker[2] *Open Journal of Physical Chemistry*, 2013, 3, 157-162 *Published Online November 2013 (http://www.scirp.org/journal/ojpc)*
http://dx.doi.org/10.4236/ojpc.2013.34019. Recently, nitric oxide (NO) has been implicated as an epigenetic factor in keloids, a scarring disease occurring primarily in dark skinned people who have relatively high amounts of pigment melanin. In this work, we tested whether a melanin-mediated redox reaction involving adsorbed NO and O2 can couple NO oxidation with O2 reduction to form reactive oxygen species (ROS) or reactive nitrogen species (RNS) *in vitro* at pH 7.4. We measured the formation of reactive species that oxidize dihydrorhodamine123 (DHR) to fluorescent rhodamine123 in the presence and absence of sepia melanin. In separate experiments, we monitored NO concentration with 4,5-diaminofluorescein (DAF) by measuring the highly fluorescent NO-adduct, DAF-2T. We attempted to detect peroxynitrite with 5 µM 3-methyl-1,2-cyclopentanedione (MCP), a selective scavenger of peroxynitrite (IC50 = 3.6 µM for $ONOO^-$ vs. 63.8 µM and >> 100 µM for NO and respectively). However, MCP itself oxidized DHR. We found that in the absence of NO, melanin itself oxidizes DHR, with no loss of DAF-fluorescence (*i.e.* no net consumption of NO). In the presence of NO, there was a ~57% loss of DAF fluorescence, indicating that NOx is formed at the expense of NO. The data provided good fit (r^2 = 0.94) to a Langmuir adsorption isotherm, with pseudo first order rate k' = 8.2 × 10^7 s^{-1}

and adsorption coefficient K_{ad} = 4.04 M^{-1}. Both of these parameters are consistent with a facile chemisorption reaction between NO and O2 on the melanin surface. Possible reactions are a) NO and O_2 → $ONOO^-$ and/or b) $2NO + O_2$ → $2NO_2$. The latter reaction is disfavored in solution but is significantly accelerated on the melanin surface via an entropy effect.

Summary of Most Important Results:

(1) We have carried out fluorescence transformation studies for the main collagen fluorophores. Most fruitful have been the 2nd order ***disappearance*** of the 270/360 nm (attributed to a disappearance of 2 like molecules; (either an eximer and/or a DOPA oxidation product) and the first order ***increase*** in 325/400 nm (attributable to dityrosine formation). The 270/360 nm species requires oxygen and is a "dark" reaction. This species accumulates on age even at 4° C. Dityrosine formation requires UV and does not accumulate in the dark. These two reactions have a "reciprocal relationship" that suggest that that the 270/360 nm species is formed at the expense of tyrosine.

(2) We have constructed Arrhenius Plots for fluorescence fading of the 270/360 nm species. Fading rates depend on the age and previous history (read "amount") of the sample. Initial lack of sensitivity to this fact resulted in data that were not reliable, and led to the hypothesis that at moderate temperatures, there was a "stable region" that reflected micro-folding regions ΔE*~ 0. In the last period, we were able to obtain better data. The activation energies (slope of the fading curve) were essentially zero from 8 – 30° C, although we could not rule out a small amount of micro-folding, since there was a slightly negative slope to the curve in this region (although it was not statistically significant). Above the denaturation point, the data reflected the random nature of the coil conformation, as it gave a *range* of ΔE* values ~ 8.8 ± 3.4 kcal/mol = 36.9 ± 14.1 kJ/mol (n = 3)

(3) We showed that melanin, ubiquitous to skin was able to couple (physiological) NO oxidation to O_2 reduction to generate ROS and RNS species. There may be a relationship between this type of chemistry and keloid development. The chemical interaction(s) among melanin, NO, O_2 and collagen remain essentially unknown.

6. LIST OF PUBLICATIONS:

(a) Papers Published in Peer – Reviewed Journals

Julian M. Menter, Comnuan Nokkaew, Danita Eatman, Aquilla Sprewell, Natalia Silvestrov, Abrienne M. Patta, Sandra Harris-Hooker (2013). The Role of Eumelanin in Generating Reactive Oxygen and Reactive Nitrogen in Solution: Possible Relevance to Keloid Formation. ***Open Journal of Physical Chemistry*, 2013, 3, 157-162** (http://www.scirp.org/journal/ojpc) http://dx.doi.org/10.4236/ojpc.2013.34019 Open Access ***OJPC***

Will be sent as separate attachment

(b) Publications in non-peer reviewed journals of in conference proceedings:

1. **Pigment melanin mediates a redox reaction between adsorbed nitric oxide and O2 in vitro J. Menter, C. Nokkaew. A. Sprewell, D. Eatman, S. Harris-Hooker**

IPCC, 2011International Pigment Cell Conference, Bordeaux, France, September 21 – 24, 2011

2. **Detection of Peroxynitrite in Melanin - NO and Melanin - Fibroblast Systems.**

Julian Menter and Comnuan Nokkaew, Morehouse School of Medicine, Atlanta GA USA

3. **DOES PIGMENT MELANIN INFLUENCE KELOID FORMATION? Menter JM, Nokkaew C, Green A, Naqvi H, and Harris-Hooker S, Morehouse School of Medicine, Atlanta GA.**

   Presented at the 2010 Meeting of the Photomedicine Society

4. **Temperature and Age – Dependence of Type I Calf Skin Collagen in vitro**
   L. Freeman, O. Edukuye, and J. Menter
   Morehouse School of Medicine, Atlanta, GA

Presented 08 July, 2012 at MSM Student Poster Day

5. **JM Menter (2014) "The Two Faces of Melanin – Protective and Anti-protective".**

Presented at the 40th meeting of the American Society for Photobiology, June 14 – 19, 2014, San Diego, CA

6. ***Drs. Julian M. Menter*, Comnuan Nokkaew, Danita Eatman, and Sandra Harris-Hooker*** **Keloids as a Model Example of Translational Research.**

Presented at BIT 4th Annual World Congress of Molecular and Cell Biology April 21 – 28, 2014 Dalian, China

7. *Effect of Temperature on Photochemical and Thermal Changes in Calf Skin Collagen Solutions at Physiological pH.*
Julian M Menter, Latoya Freeman and Otega Edukuye, Morehouse School of Medicine, Atlanta, GA 30310-1495

To be presented at the BIT Molecular Medicine Conference Haikou, Hainan Province, P.R.China.

N.B. Only the last publication is directly concerned with the temperature – dependent behavior of ground- and excited state behavior of collagen in ground and excited states. This is because only in the last several months have we obtained data that is precise enough to publish. This is all explained (hopefully) in sufficient detailed progress report.

We do plan on submitting one or more manuscripts outlining our results in the not – too – distant future. We will keep you abreast of the situation as it develops.
JMM

d. MANUSCRIPTS SUBMITTED BUT NOT PUBLISHED: NA

e. TECHNICAL REPORTS SUBMITTED TO ARO : NA

7. LIST OF PARTICIPATING SCIENCE PERSONNEL:

(1) Julian M Menter, PhD – Principal Investigator
(2) Abrienne M Patta, BS - MPH
(3) Comnuan Nokkaew PhD – Co-investigator
(4) Sandra Harris – Hooker PhD – Co-investigator
(5) LaToya Freeman – Medical Student
(6) Otega Edukuye – Medical Student

8. LIST OF INVENTIONS: NA

9. BIBLIOGRAPHY:

(1) JM Menter, Comnuan Nokkaew, Danita Eatmanm Aquilla Sprewell, Natalia Silvestrov, Abrienne M Patta, Sandra Harris - Hooker **(2013)The Role of Eumelanin in Generating Reactive Oxygen and Reactive Nitrogen in Solution: Possible Relevance to Keloid Formation *Open Journal of Physical Chemistry*, 2013, 3, 157-162** Published Online November 2013 (http://www.scirp.org/journal/ojpc) http://dx.doi.org/10.4236/ojpc.2013.34019

(2) JM Menter Danita Eatman, Mohamed Bayorh, Ahmad M. Dawaghreh, and Isaac Willis **(2008) Pigment Melanin Scavenges Nitric Oxide In Vitro: Possible Relevance to Keloid Formation *Research Letters in Physical Chemistry* Volume (2008), Article ID 210616, 4 pages doi:10.1155/2008/210616)**

(3) JM Menter, *Photochem. Photobiol. Sci*. ***(2006): 5, 403–410 DOI: 10.1039/b516429j.***

(4) Karl E. Kadler, Yoshio Hojima, and Darwin Prockop **(1988) Assembly of Type I Collagen Fibrils *de Novo*. Between 37 and 41 ^{o}C the process is limited by micro-unfolding of monomers. *J Biol. Chem. 263(21) 10517 – 1063***

10. APPENDIX – SEE ABOVE.

APPENDIX

-C5~r

Pigment melanin mediates a redox reaction between adsorbed nitric oxide and O_2 in vitro

J. Menter, C. Nokkaew. A. Sprewell and D. Eatman,
S. Harris-Hooker

Morehouse School or Medicine. Atlanta, GA. USA

Pigment melanin can adsorb molecular O_2• scavenge nitric oxide (NO) and thereby coupie a redox reaction betvveen them. in this work. we show formation of peroxynitrite (ONOO-) in the presence but not in the absence of melanin. NO generated by DENNO or SNAP *vvas* dialyzed into membranes containIng purified sepia melanin in 0.1 M phosphate buffer, pH 7.4 or control buffer alone. NO was measured as nitrite and nitrate 'via the Greiss methodology and *by* the DAf fluorescence assay. Peroxynitrite vvas detected *by* selective scavenging with 3.3 fLM MCP or via detection of ni1rotyrosine in cultured fibroblasts. H:P2 w-'oS monitored by the scopoletirJperoxidase assay. Appropriate controls were used. Dialyzate NO concentrations were significantly lower in the test dialyzates than in controls. In the test systems in vitro we detected significant amounts of peroxynitrite but little Of no hydrogen peroxide. No significant amounts of either of these were detected in the absence of melanin. In cultured iibroblasts, we observed positive staining for nitrotyrosine in the presence, but not in the absence of melanin. Sepia melanin can couple the redox reaction between adsorbed NO ana O_2 to afford ONOO - via a superoxide intermediate. Superoxide can undergo 'pseudodismutation' to H_20_2 and O_2 by melanin or reaction with NO. Peroxide is scavenged by melanin, and is not detected in significant amounts. Supported in Part by MBRS Grant ifGM 08248, RCMl Grant ifRR 03034 and DOD Grant *it* W911 NF - 10 - 1 - 0448.

Detection of Peroxynitrite in Melanin - NO and Melanin - Fibroblast Systems.

Julian Menter and Comnuan Nokkaew, Morehouse School of Medicine, Atlanta GA USA

BACKGROUND: Recently, nitric oxide (NO) has been implicated in the formation of keloids, a scarring disease resulting from abnormal wound healing of skin. Last year, we demonstrated the ability of cuttlefish sepia melanin (Melco) to scavenge donor generated NO through a dialysis membrane in vitro. As we could not account for the added NO (as nitrate and nitrite) in the system that contained added melanin, we suspected the formation of reactive nitrogen species (RNS), e.g. peroxynitrite, may have been formed by reaction of NO with or in the presence of added melanin.

METHODS: Melanin Preparation: Purified sepia melanin (MelanInk@, Vacaville CA) extracted from cuttlefish was pre dialyzed through a Spectropore membrane (MW cutoff 6-8 kD) into 100 mL 0.1 M phosphate buffer, pH 7.4/0.1 M EDTA, followed by 2 changes of 0.1 M buffer alone.

Measurement of NO: NO was measured as nitrite and nitrate with a SOFT maxPRO NO-measuring kit (Molecular Devices) using the manufacturers instructions.

Generation of NO: The NO-generating compounds, 2-(N.N Diethylamino) diazeneolate -2 oxide (DEA/NO) or S nitroso N acetylpenicillamine (SNAP) in phosphate buffer were used. Dialyzates were analyzed for NO. Control dialyses were run in the absence of melanin.

Detection of peroxynitrite: ONOO- was detected by selective scavenging with 3-methyl - 1, 2 cyclopentanedione (MCP). Dialysis systems contained 10 mg sepia melanin, 1.0 mg SNAP ± 250 ¼l MCP (total [MCP] = 3.3 ¼M). Test: Melanin + MCP + SNAP. Controls: SNAP alone; SNAP + MCP; Melanin alone; Melanin + MCP

RESULTS: In experiments in vitro with the peroxynitrite scavenger 3.3 µM 3 methyl 1,2 cyclopentanedione (MCP), we detected significant amounts of peroxynitrite in the test systems (added melanin) but not in the controls (melanin - free) In experiments with human fibroblasts in tissue culture, we found that addition of soluble melanin (Melco) to the fibroblast culture system followed by incubation at 37 C resulted in formation of 3-nitrotyrosine, (a sign of the presence of peroxynitrite) whereas the melanin free system showed no such nitration.

CONCLUSIONS: These results indicate that melanin can mediate a reaction between NO and (probably) superoxide to form peroxynitrite, a potent cytotoxic compound. This finding may indicate a melanin mediated mechanism by which NO up-regulates fibroblast collagen production via formation of cytotoxic peroxynitrite.

Supported in part by MBRS Grant #GM08248 and RCMI Grant #RR 03034. Conflicts of interest: none

DOES PIGMENT MELANIN INFLUENCE KELOID FORMATION? **Menter JM, Nokkaew C, Green A, Naqvi H, and Harris-Hooker S, Morehouse School of Medicine, Atlanta GA.**

Purpose: Nitric oxide (NO) has been implicated in the formation of keloids. As keloids preferentially occur in Blacks, pigment melanin might be involved. Cuttlefish sepia melanin can scavenge donor - generated NO through a dialysis membrane *in vitro*. We hypothesize that reactive nitrogen (RNS) and/or reactive oxygen species (ROS) may occur from melanin – mediated NO oxidation.

Design: Purified sepia melanin extracted from cuttlefish was used. NO was generated in vitro with 0.100 mM 2-(N.N Diethylamino) S - nitroso - N - acetylpenicillamine (SNAP) in phosphate buffer. It was assessed by measurement of its fluorescent adduct with 4, 5 - diaminofluorescein (DAF). Peroxynitrite ($ONOO^-$) was generated in vitro via 0.250 mM 3 – morpholino – syndonimine (SIN – 1), and assessed with the selective scavenger 3.3 μM 3 - methyl - 1, 2 - cyclopentanedione (MCP) in solution. We incubated cultured human adult dermal fibroblasts with and without 0.1 mM melanin, and tested for peroxynitrite via Western and immunocytofluorometric assays using a 3- nitrotyrosine antibody. H_2O_2 was determined via the scopoletin assay.

Results: We detected significant amounts of peroxynitrite in melanin – containing systems, but not in controls. We could detect little or no H_2O_2 in these systems. Addition of soluble melanin to the fibroblast culture system resulted in formation of 3-nitrotyrosine. Comparison of the test and control systems by immunocytofluorometry and by Western blotting indicated a small but significant amount of 3-nitrotyrosine in the test cultures. At high 3-nitrotyrosine levels, melanin conferred significant protection from tyrosine nitration.

Conclusions: These results indicate melanin's chemically complex nature where both protective and cytotoxic effects to fibroblasts can be observed in concentration – dependent manner. **Peroxynitrite** is produced by redox reactions involving melanin, **superoxide,** and **NO**. **Peroxide** may arise from dismutation of **superoxide** by melanin, but it will be **re-scavenged** by melanin. Peroxynitrite may therefore play a significant role in keloid formation. **Supported by MBRS Grant #GM08248 , RCMI Grant #RR 03034 and DOD Grant # W911 NF- 10- 1- 0448.**

Presented at the 2010 Meeting of the Photomedicine Society

Temperature and Age –
Dependence of Type I Calf Skin Collagen in vitro
L. Freeman, O. Edukuye, and J. Menter
Morehouse School of Medicine, Atlanta, GA
Presented 08 July, 2012

(c) Presentations

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Title:

"The Two Faces of Melanin – Protective and Anti-protective".

Abstract: (Your abstract must use Normal style and must fit into the box. Do not enter author details)

" Melanin" refers to a *group* of pigments, Eumelanin is thought to comprise numerous cross-linked 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) polymers, Pheomelanin differs from eumelanin in that its oligomer structure incorporates benzothiazine and benzothiazole units that are produced instead of DHI and DHICA. Neuromelanin is formed from catecholamine oxidation pathways. All of these melanins possess broad featureless absorption bands, can act as semiconductors, can bind metals and organic material (drugs) and act as free radical scavengers. These properties confer the ability of melanin to act simultaneously as a protector and/or as a sensitizer. For example, melanin sequestration of drugs or metals can protect vulnerable cells or tissue from deleterious effects by these agents. On the other hand, bound transition metals (e.g. iron) can lead to formation of harmful reactive oxygen or nitrogen species. The particular chemistry of melanin can influence the relative importance of protective vs. anti-protective behavior to solar radiation. Eumelanin is generally thought to be photo-protective, while pheomelanin is a photosensitizer. Neuromelanin can bind large of iron and is thought to play a role in iron homeostasis. However under iron overload it could play a toxic role by promoting redox reactions. Extensive electron delocalization stabilizes melanin radicals but also allows melanin "mediate" potentially harmful redox reactions between electron donor and acceptor molecules adsorbed to the melanin backbone.

We have previously demonstrated that synthetic dopa-melanin and sepia melanin can couple the oxidation of catecholic skin depigmenters to potassium ferricyanide reduction *in vitro.* More recently, we have shown that co-adsorbed nitric oxide (NO) and molecular O_2 will react to form reactive nitrogen species (RNS), most likely $ONOO^-$ and NO_2 at rates much faster than would occur in the absence of melanin pigment. This latter observation is of significance to keloid pathology, since NO is known to up-regulate type I collagen in humans, and since keloid scarring is observed preferentially in darkly – pigmented persons. **Funded in part by GRANTS: MBRS #GM08248, RCMI #8G12MD007602, and DOD # 911 NF – 10 – 1 0448. There are no conflicts of interest.**

Presented at the 40th meeting of the American Society for Photobiology, June 14 – 19, 2014, San Diego, CA

Title: Keloids as a Model Example of Translational Research.

Drs. Julian M. Menter*, Comnuan Nokkaew, Danita Eatman, and Sandra Harris-Hooker

Research Professor

Morehouse School of Medicine

USA

Abstract

People of color are particularly susceptible to *keloids*, a recalcitrant consequence of aberrant wound healing characterized by excessive collagen deposition. Although many studies have sought to elucidate the pathological causes, no clear picture has emerged. There are no good animal models because keloids occur mainly in humans. A promising recent approach to addressing this problem is use of tissue engineering techniques to generate a stable 3-dimensional keloid cell culture system that can be grafted onto nude mice. This technique can allow systematic studies of promising therapies and/or pathology of aberrant tissue in humans. In a cell-free system, we demonstrated that pigment melanin mediates a redox reaction between co-adsorbed nitric oxide and molecular O_2 to afford toxic RNS and ROS species that could up-regulate deposition of connective tissue matrix. Comparison of normal to abnormal fibroblasts is now possible. **Supported by MBRS #GM08248, RCMI #03034, and DOD# W911-NF-10-1-448 Grants .**

Biography

Dr. Menter received his Ph.D. degree in Chemistry from the George Washington University in 1969. He completed a postdoctoral fellowship with Prof. Dr. Theodor Foerster at the Institut fuer physikalische Chemie der Universtiaet Stuttgart, Germany where he documented the first example of an adiabatic photochemical reaction in which a stable photoproduct appears in the excited state. Following his post doctoral fellowship, he joined the faculty of Engineering Biophysics at the University of Alabama at Birmingham from 1971 - 1978, the VA Medical Center (Atlanta) from 1978 - 1982.He currently serve as Research Professor of Microbiology, Biochemistry and Immunology at Morehouse school of Medicine. Dr. Menter has accumulated more than 100 scientific publications. He is recognized internationally for his work in the areas of collagen photochemistry and photobiology, and the redox properties of pigment melanin, as relating to nitric oxide chemistry.

Presented at BIT 4th Annual World Congress of Molecular and Cell Biology April 21 – 28, 2014 Dalian, China

Title: *Effect of Temperature on Photochemical and Thermal Changes in Calf Skin Collagen Solutions at Physiological pH.*

Julian M Menter, Latoya Freeman and Otega Edukuye, Morehouse School of Medicine, Atlanta, GA 30310-1495

Abstract

Mammalian collagens contain several age-related fluorescent chromophores derived from (photo)oxidation of tyrosine residues and/or glycation of free amino acid. Since these compounds may be photosensitizers or otherwise deleterious, it is important to know the chemical properties as well as the effects of temperature and the presence of oxygen on forming these age-related compounds under physiological conditions. Fluorescence be observed, whereas other properties (e.g. electrophoresis, appearance) may not be sensitive to age and temperature, probably because these fluorophores form a very small proportion of the collagen molecule. Deviation of Arrhenius plots from linearity suggests a change of phase and/or the simultaneous presence of more than one reaction. The non-linear plot observed in *figure 3* is consistent with the collagen's helix-coil transition. Above T_m, the slopes of the measured fluorescence intensities are uncertain owing to the essentially random orientation so that estimated activation energies and values of T_m must be viewed as approximations. The data indicate that there is little or no activation involved in the photochemistry of the helical structure, There may be a small negative activation energy (*fig 3B*), indicating a possible "stable" region due to micro-unfolding near T_m (*K. Kadler et al, J Biol. Chem. 263:10516 – 10523, 1998).*The reciprocal relationship between the rate of 270/360 nm photo-degradation and consequent formation of stable dityrosine (excitation /emission at 325/400 nm) indicates that the two processes are interconnected. The 270/360 nm emission species appears to be a "double molecule". We have previously postulated this species to be due to an excimer - like interaction between two molecules in close proximity. However it could be due to covalent di-DOPA cross-links that disappear by UV- mediated formation of a non-fluorescent product. Our collagen samples were very hygroscopic and it was not possible for us to completely remove H_2O. This allowed "dark" oxidation of tyrosine to DOPA. Our data thus indicate that di-DOPA may form via thermal oxidation of tyrosine at the latter's expense. **This work was supported in part by DOD Grant # 911 NF-10-1, MBRS Grant # GM08248, and RCMI Grant # 8G12MD00760**

To be presented at the BIT Molecular Medicine Conference Haikou, Hainan Province, P.R.China.

Peer – Reviewed Manuscript;

Julian M. Menter, Comnuan Nokkaew, Danita Eatman, Aquilla Sprewell1, Natalia Silvestrov Abrienne M. Patta, Sandra Harris-Hooker "The Role of Eumelanin in Generating Reactive Oxygen and Reactive Nitrogen in Solution: Possible Relevance to Keloid Formation" ***Open Journal of Physical Chemistry*****, 2013, 3, 157-162** Published Online November 2013 (http://www.scirp.org/journal/ojpc) http://dx.doi.org/10.4236/ojpc.2013.34019 Open Access ***OJPC***

Acknowledgements This work was funded in part by GRANTS: MBRS #GM08248, RCMI #8G12MD007602, and DOD # 911 NF-10-1 0448. There are no conflicts of interest.

This manuscript will be sent as a separate attachment.

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DD FORM 882

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

Form Approved
OMB No. 9000-0095
Expires Jan 31, 2008

PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THE ABOVE ORGANIZATION. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.

1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT *(X one)*
Morehouse School of Medicine, Inc.	W911NF-10-1-0448	Same as Item 1.a.		a. INTERIM [ ] b. FINAL [X]
b. ADDRESS *(Include ZIP Code)*	**d. AWARD DATE** *(YYYYMMDD)*	**b. ADDRESS** *(Include ZIP Code)*	**d. AWARD DATE** *(YYYYMMDD)*	**4. REPORTING PERIOD** *(YYYYMMDD)*
720 WESTVIEW DRIVE, S.W. ATLANTA, GA 30310-1495	20100913			a. FROM 20100913 b. TO 20140912

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
NONE								

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*	(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME OF SUBCONTRACTOR(S) a.	ADDRESS *(Include ZIP Code)* b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES *(YYYYMMDD)* f.	
			(1) CLAUSE NUMBER	(2) DATE *(YYYYMM)*		(1) AWARD	(2) ESTIMATED COMPLETION
NONE							

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))* [ ] SMALL BUSINESS or [X] NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Harris-Hooker, Sandra	Vice President and Vice Executive Dean for Research and Academic Administration	Sandra Harris-Hooker	20141013

SF425

FEDERAL FINANCIAL REPORT

1. Federal Agency and Organizational Element to Which Report is Submitted	2. Federal Grant or Other Identifying Number Assigned by Federal Agency (To report multiple grants, use FFR Attachment)	Page	of
US Army/ONRRO	W911NF-10-1-0448	1	1 pages

3. Recipient Organization (Name and complete address including Zip code)

Morehouse School of Medicine, 720 Westview Drive, SW, Atlanta, GA 30310

4a. DUNS Number	4b. EIN	5. Recipient Account Number or Identifying Number (To report multiple grants, use FFR Attachment)	6. Report Type	7. Basis of Accounting
102005451	58-1438873	215011	☐ Quarterly ☐ Semi-Annual ☐ Annual X Final	☐ Cash ☑ Accrual

8. Project/Grant Period From: (Month, Day, Year)	To: (Month, Day, Year)	9. Reporting Period End Date (Month, Day, Year)
9/13/2010	9/12/2014	9/12/2014

10. Transactions	Cumulative
(Use lines a-c for single or multiple grant reporting)	
Federal Cash (To report multiple grants, also use FFR Attachment):	
a. Cash Receipts	$0.00
b. Cash Disbursements	$0.00
c. Cash on Hand (line a minus b)	$0.00
(Use lines d-o for single grant reporting)	
Federal Expenditures and Unobligated Balance:	
d. Total Federal funds authorized	$350,371.00
e. Federal share of expenditures	$338,330.53
f. Federal share of unliquidated obligations	
g. Total Federal share (sum of lines e and f)	$338,330.53
h. Unobligated balance of Federal funds (line d minus g)	$12,040.47
Recipient Share:	
i. Total recipient share required	
j. Recipient share of expenditures	
k. Remaining recipient share to be provided (line i minus j)	$0.00
Program Income:	
l. Total Federal program income earned	
m. Program income expended in accordance with the deduction alternative	
n. Program income expended in accordance with the addition alternative	
o. Unexpended program income (line l minus line m or line n)	$0.00

11. Indirect Expense	a. Type	b. Rate	c. Period From	Period To	d. Base	e. Amount Charged	f. Federal Share
	Pre-Determined	41.50%	09/13/10	09/12/14	232,993.50	96,692.29	96,692.29
				g. Totals:	$232,993.50	$96,692.29	$96,692.29

12. *Remarks: Attach any explanations deemed necessary or information required by Federal sponsoring agency in compliance with governing legislation:*

13. Certification: By signing this report, I certify that it is true, complete, and accurate to the best of my knowledge. I am aware that13. Certification: By signing this report, I certify that it is true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent information may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 218, Section 1001) any false, fictitious, or fraudulent information may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 218, Section 1001)

a. Typed or Printed Name and Title of Authorized Certifying Official	c. Telephone (Area code, number and extension)
Sherry A. Ballenger, Director, Office of Grants and Contracts	404-752-1546
	d. Email address: sballenger@msm.edu
b. Signature of Authorized Certifying Official	e. Date Report Submitted (Month, Day, Year): 12/11/14
	14. Agency use only:

Standard Form 425
OMB Approval Number: 0348-0061
Expiration Date: 10/31/2011

Paperwork Burden Statement

According to the Paperwork Reduction Act, as amended, no persons are required to respond to a collection of information unless it displays a valid OMB Control Number. The valid OMB control number for this information collection is 0348-0061. Public reporting burden for this collection of information is estimated to average 1.5 hours per response, including time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding the burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to the Office of Management and Budget, Paperwork Reduction Project (0348-0061), Washington, DC 20503.